Porcine reproductive and respiratory syndrome virus (PRRSV) in pig meat

Philippe Raymond, Christian Bellehumeur, Malliga Nagarajan, Diane Longtin, Alexandra Ferland, Peter Müller, Rachel Bissonnette, Carole Simard

Abstract

Porcine reproductive and respiratory syndrome, caused by the porcine reproductive and respiratory syndrome virus (PRRSV), is an economically important disease in the swine industry. Previous studies demonstrated the presence of the virus in pig meat and its transmissibility by oral consumption. This study further analyzed the infectivity of PRRSV in commercial pig meat. Fresh bottom meat pieces (n = 1500) randomly selected over a period of 2 y from a pork ham boning plant located in Quebec, Canada, were tested by reverse transcriptase polymerase chain reaction (RT-PCR). Each trimmed meat was stored in the plant freezer, subsampled weekly for up to 15 wk, and tested with quantitative RT-PCR to determine the viral load. Meat infectivity was evaluated using specific pathogen-free piglets, each fed with approximately 500 g of meat at the end of the storage time. Genotype-specific RT-PCR confirmed the presence of PRRSV mainly during cold weather in 0.73% of the fresh meat pieces. Wild and vaccine strains of genotype 2 were detected. Porcine reproductive and respiratory syndrome virus nucleic acid was stable in meat stored at around −20°C during the 15 wk. Serological and molecular analysis showed the transmission of infection by a majority of PRRSV positive meat pieces (5/9) fed orally to naïve recipients. The results confirmed a low prevalence of PRRSV in market's pig meat, and virus transmissibility by oral consumption to naïve recipients even after several weeks of storage in a commercial freezer. It occurred mainly with meat harboring the highest PRRSV RNA copies, in the range of 10^9 copies per 500 g of meat, with both wild type and vaccine-related strains.

Résumé

Le syndrome reproducteur et respiratoire porcin, causé par le virus du syndrome respiratoire et reproducteur porcin (vSRRP), est une maladie ayant un impact économique important pour l'industrie porcine. Des études antérieures ont démontré la présence du virus dans la viande de porc ainsi que sa transmissibilité par ingestion. La présente étude poursuit l'analyse de l'infectiosité du vSRRP dans la viande commerciale de porc. Des coupes de fesses de porc fraîches (n = 1500) sélectionnées aléatoirement sur une période de deux ans dans une usine de désossage située au Québec (Canada), ont été testées en utilisant une transcription réverse suivie d'une amplification en chaîne par polymérase (RT-PCR). Chaque pièce de viande parée a été entreposée dans les congélateurs à l'usine, échantillonnée hebdomadairement pendant 15 semaines, et testée par RT-PCR quantitatif afin de calculer la charge virale. Le potentiel infectieux a été évalué sur des porcelets exempts d'agent pathogène spécifique qui ont été nourris avec approximativement 500 g de viande à la fin de la période d'entreposage. Une RT-PCR spécifique au génotype a confirmé la présence du vSRRP principalement durant les temps froids, dans 0,73 % des pièces de viandes fraîches. Des souches sauvages et vaccinales du génotype 2 ont été détectées. L'acide nucléique du virus du syndrome respiratoire et reproducteur porcin est demeuré stable dans la viande durant la période d'entreposage de 15 semaines à −20 °C. L'analyse sérologique et moléculaire a démontré la transmission de l'infection par une majorité des pièces de viande positives au vSRRP (5/9) chez les porcelets naïfs ayant consommé la viande. Les résultats confirment la faible prévalence du vSRRP dans la viande distribuée sur le marché ainsi que la transmissibilité du virus par consommation orale chez des hôtes naïfs même après plusieurs semaines d'entreposage dans un congélateur commercial. La transmission s'est produite surtout avec les viandes ayant un nombre de copies d'ARN de vSRRP plus élevés, environ 10^9 copies par 500 g de viande, associées à des souches de type tant sauvage que vaccinal.

(Traduit par les auteurs)

Introduction

Porcine reproductive and respiratory syndrome (PRRS) is among the most economically significant swine infectious diseases (1,2). The role of pig meat and swill feeding in PRRS virus (PRRSV) transmission is questioned as international trade in pork expands (3). The enveloped virus is between 50 and 65 nm in diameter and is classified in the *Arteriviridae* family within the order *Nidovirales*. It contains a single-stranded positive-sense RNA of approximately 15 kb in length that encodes at least 9 open reading frames (ORF) (4). There are 2 recognized PRRSV genotypes: the North American (genotype 2, VR-2332 prototype) and the European (genotype 1, Lelystad prototype). Both have similar genomic organizations, but are genetically and antigenically distinct (5). In Canada, the North American genotype has been reported in lineages typical of vaccines (Ingelvac PRRS ATP, Ingelvac PRRS MLV) and of the MN-184 wild types (6,7). Porcine reproductive and respiratory syndrome is mainly characterized by reproductive failure in sows and respiratory

Canadian Food Inspection Agency, Saint-Hyacinthe Laboratory, Saint-Hyacinthe, Quebec J2S 8E3.

Address all correspondence to Dr. Philippe Raymond; telephone: (450) 768-6808; fax: (450) 768-6767; e-mail: philippe.raymond@inspection.gc.ca

Received July 7, 2016. Accepted January 17, 2017.

illness in pigs of all ages. These clinical signs vary markedly between herds and depend on the virulence of the infecting strain. In eastern Europe and China, new highly pathogenic subtypes of PRRSV have been reported (8–10).

Porcine reproductive and respiratory syndrome virus is highly contagious in pig herds. It can be transmitted by direct contact or by infectious aerosols. Vaccines have been used to limit the propagation of the disease, but are often found inefficient (11). The virus can also persist for months in the target cells of the monocyte and macrophage lineages of an infected animal, despite the immune protection (11,12). Transient detection of PRRSV in pig meat following experimental transmission has been observed (12). The virus was isolated *in vitro* from 6 out of 1049 sample pools of fresh meat (13), while others failed to detect PRRSV in 472 pig carcasses tested by PCR (14).

In countries currently free of PRRS, the risk of importing the PRRS virus in fresh pork is a concern. Some early import risk analyses for chilled or frozen meats have concluded that virally infected pig meat could represent a source for the introduction of the virus in PRRS-free countries (15,16). These analyses were notably based on a study from Lelystad, the Netherlands, showing the transmission of PRRSV through oral uptake of infected porcine muscular tissues by naïve recipients (17,18). In 2003, Magar and Larochelle (6) led a study to investigate whether pig meat could harbor PRRSV and if so, whether viruses in positive meats could infect the animals when fed to SPF pigs. In their study, analyses and bioassays were performed with meat conserved at ultra-low temperatures. It was argued that these experiments were performed in optimal conditions for virus survival in meat (19–21). For instance, van der Linden et al (18) showed that freezing meat at −23°C for 10 d and then thawing it decreased the virus titers in the majority of PRRSV infected muscle samples. The present study was undertaken to reproduce conditions in commercial settings under which meat would be cut, packaged, and stored in a frozen state for many weeks, similar to overseas import or export conditions. It provides additional knowledge on PRRSV prevalence, survival in pig meat, meat viral load, and transmission from meat to naïve piglets.

Materials and methods

Pork samples

Pork carcasses were from market pigs killed at 3 abattoirs from the province of Quebec, Canada. Carcasses were transported in refrigerated trucks and received at a pork ham boning plant (PHBP) located in St. Hyacinthe, Quebec, 2 to 5 d after slaughter. Between 5 and 17 fresh bottom meat pieces of around 900 to 1100 g each were selected randomly from the cutting and trimming lines 2 to 3 d a week (117 visits). The meat pieces could not be associated with a specific abattoir once on the cutting and trimming line. Each meat piece was identified using a unique identification number (ID). Approximately 2 g of muscle tissue was subsampled from each meat piece with a sterile scalpel at 3 distant locations and pooled in a 50 mL sterile tube. These subsamples corresponded to week 0 (T_0). Meat subsamples were kept on ice until their arrival at the CFIA Saint Hyacinthe Laboratory for RNA extraction a few hours later. At the PHBP, each of the selected fresh bottom meat pieces was put in a labeled plastic bag and stored immediately in cardboard boxes among commercial meats in the company's freezer, maintained between −24°C and −21°C. Based on the reverse transcriptase polymerase chain reaction (RT-PCR) assays, 1 PRRSV non-confirmed, 9 PRRSV positive, and 2 RT-PCR PRRSV negative frozen meat pieces were selected for further analysis. In storage follow-up experiments, they were subsampled weekly at the PHBP from T_1 up to 15 consecutive weeks (T_{max}). Subsamples were collected in each frozen meat piece near the first sampling locations using a clean and sterilized bit and a drill. Approximately 2 g of frozen meat were subsampled and kept on ice until processed at the lab a few hours later. The bottom frozen meat pieces were immediately put back in their boxes in the company's freezer, among commercial meats. At the last sampling time (T_{max}), leftover frozen meats were transported in a frozen state and stored at −70°C, until used in viral transmission bioassays.

Viral transmission bioassays

All animals were treated according to the policy and guidelines of the Canadian Council on Animal Care (CCAC) and the Animal Care Committee of our laboratory. For the feeding trials, 9 PRRSV RT-PCR confirmed positive and one non-confirmed pig meat sample were fed to the piglets. These bioassays were adapted from Magar and Larochelle (6) using the same facilities, biosecurity measures, and animal adaption protocol. Each meat sample was used to feed 2 specific pathogen-free (SPF) piglets 5 to 6 wk of age. Each pig pair was housed in a separate cubicle. These piglets were provided by the CFIA Ottawa Laboratory (Fallowfield, Ontario). The meat samples were thawed at 4°C overnight, weighed, cut into small pieces (around 2 cm^3) with sterile scalpels, and divided in 4 equal portions of approximately 250 g each. Piglets were fed a portion on 2 consecutive days. The portion used to feed the pigs on the second day was kept at 4°C overnight. In an alternative protocol, 3 meat samples (ID 1311, 1332, and 1424) were also divided into 4 equal portions of around 250 g. While 2 portions were stored at 4°C overnight, the other 2 were stored at room temperature (RT, between 18°C to 23°C). A pig was fed on 2 consecutive days with the meat sample stored at 4°C while the paired animal, located in a separate cubicle, received the other part of the same meat sample stored at RT. Each trial included either 1 or 2 control pigs maintained on a standard pig diet with no thawed meat throughout the experiments.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected on a regular basis from the jugular vein of each piglet with a 20-G needle. Throat mucus samples were collected with minitip flocked swabs (Millipore; Billerica, Massachusetts, USA) to follow PRRSV infection in piglets. Both were collected at arrival and at 0, 7, 14, 21, and 28 d after feeding. Only the throat samples from the first 3 bioassays were tested. Serum and throat samples were used to detect PRRSV by molecular assays. Serum samples were tested for the presence of antibodies to PRRSV in piglets via ELISA (HerdCheck; IDEXX Laboratories, Westbrook, Maine, USA) as recommended by the manufacturer.

RNA extraction

All meat subsamples from the PHBP were homogenized on the same collecting day as previously described (22). Supernatant

aliquots were stored at −70°C until nucleic acid extraction. RNA was extracted from 100 μL of the meat homogenate using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Total RNA was eluted with 40 μL of RNase-free water. A Nanodrop-1000 (Thermo Scientific, Wilmington, Delaware, USA) was used to quantify total RNA in each sample (OD 260 nM). For the meat-fed piglets, RNA was extracted from 140 μL of serum sample using the QIAamp Viral RNA Mini Kit (Qiagen). RNA was also extracted from throat swab samples using RNeasy Mini Kit with 0.6 mL RLT buffer. All RNA extracts were stored at −70°C prior to the analyses. The OR7 RT-PCR analyses were conducted within a week while samples from decay were combined on the same plate and tested by OR7 qRT-PCR at the end of the storage period. Phylogeny analyses were conducted on RNA extract stored up to 3 y.

RT-PCR

The RT-PCR for the detection of the ORF-7 gene of both American and European strains was performed on 1500 fresh meat samples collected randomly as a screen test (T_0). A 3 μL volume of RNA extract was mixed with 0.48 μM of primers P1 and P2 developed previously and tested using the Qiagen One-Step RT-PCR Kit and the GeneAmp PCR System 9770 (Applied Biosystems, Carlsbad, California, USA) in the presence of RNasin (Promega, Madison, Wisconsin, USA) (23). The RT-PCR included a reverse transcription step at 50°C for 30 min and a PCR amplification step with enzyme activation at 95°C for 15 min followed by 34 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30s, and a final elongation step at 72°C for 7 min. When analyzed on ethidium bromide agarose gel (2%) a specific band of 303 bp was expected for genotype 2, whereas a band of 291 bp was expected for the genotype 1. A sample having a band of the expected molecular weight was declared PRRSV presumptive, if the positive and negative controls were valid. Each presumptive positive RNA sample was retested with distinct ORF-7 RT-PCR using similar RT-PCR mix and amplification conditions, but different primer sets. These tests were also done to determine the genotype of the virus. The primer set PR15M (GGTAAGATCATCGCTCAGCA) and PR16M (GACACAATTGCCGCTCACTA) was developed in this study and was specific to genotype 2 (ORF-7 RT-PCR) (data not shown). This primer set amplified a region that corresponds to bp position 14969 to 15116 of NVSL (AY545985.1). To detect genotype 1, P4 and P2 primers were used (23). Each presumptive positive was also tested with PRRSV primers UN17F and UNI17R used in the study of Magar and Larochelle (6). A presumptive meat was confirmed PRRSV positive using at least 2 distinct extracts tested with ORF-7 RT-PCR assays, otherwise it was declared non-confirmed. This approach was used to reduce the number of false positives. Meat harboring a virus level close to the LOD might have been classified as non-confirmed in this process as well as some cross-contaminated pieces.

RNA transcript

An RNA transcript was produced from the PRRSV NVSL strain 97-7895 using the PR29F (TTAATACGACTCACTATAGGGTGGG TAAGATCATCGCTCAGCA) and PR30R (TTGACGACAGACA CAATTGCCGCT) primer set as well as the MEGAshortscript T7 Kit, and purified with the MEGAclear Kit (Applied Biosystems/Ambion, Mississauga, Ontario). The RNA transcript was quantified with the Nanodrop-1000.

SYBR Green quantitative Real-Time RT-PCR (qRT-PCR)

To assess viral load before the transmission experiment at T_{10} or T_{max} in meats and to evaluate viral decay over time from T_1 to T_{max}, viral RNA copies were estimated by one-step SYBR Green qRT-PCR assay (ORF-7 qRT-PCR) using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) an the RNA transcript standard. The PR15M and PR16M primers and the MX4000 or MX3005p real-time thermocyclers (Agilent Technologies, Santa Clara, California, USA) were used for these assays. The qRT-PCR included a reverse transcription step at 50°C for 30 min and a PCR amplification step with enzyme activation at 95°C for 15 min. These were followed by 40 cycles of 95°C for 15 s, 57°C for 30 s, and 72°C for 30 s, and finally a dissociation cycle. All samples from the same animal (T_0 to T_{max}) were analyzed on the same plate to avoid inter-assay variability. In the presence of a specific melting temperature [Mt (78.5°C to 80.5°C)], the sample was declared positive. Samples were declared positive but not quantifiable for PRRSV when non-specific Mt was detected in addition to the specific one. These results were indicative of a simultaneous non-specific amplification and were detected only below 100 viral RNA copies/μL, which represents the limit of quantification (LOQ). No specific amplification was detected more than 95% of the time below the limit of detection (LOD) of 10 viral RNA copies/μL. The number of viral RNA copies per mg of each quantifiable subsample tested by qRT-PCR was calculated according to the standard curve multiplied by the RNA extract volume (40 μL), divided by the extraction efficacy factor (7%) and by the weight of the meat homogenate sample (20 mg per extract), and normalized using the total RNA extracted.

Porcine circovirus (PCV) detection

For the detection of PCV nucleic acid in meat extract, DNA was extracted using the DNeasy Tissue Kit (Qiagen) and recovered with 50 μL of elution buffer. A volume of 3 μL was tested using the Platinum *Taq* DNA Polymerase Kit (Invitrogen), 1.5 mM MgCl2, and 0.2 μM of each VCP5F (AGTGAGCGGGAAAATGCA) and VCP6R (CACACAGTCTCAGTAGATCATCC) primers. This primer set amplified a region that corresponds to bp position 515 to 741 of postweaning multisystemic wasting syndrome PCV (AF027217.1). The PCR was achieved after 2 min of incubation at 95°C followed by 35 cycles at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min. The PCR products were kept at 4°C until agarose gel evaluation. Positive results for the PCR were further confirmed using the same PCR conditions with primer set VCP22F (TGGCCCGCAGTATTCTGATT) and VCP23R (CAGCTGGGACAGCAGTTGAG) that amplified a region that correspond to bp position 790 and 861 of postweaning multisystemic wasting syndrome PCV.

Virus decay

To estimate virus decay over time, a ratio of viral RNA copy (C_x/C_1) was calculated for each quantifiable subsample of a storage follow-up series, where C_x represents the copy numbers/μL for a specific subsample from week 2 to week 10 (T_x), and C_1 represents the value obtained at week one (T_1). The degradation rate "k" was

determined by plotting the natural logarithm transformation of the ratio of viral RNA copies (C_x/C_1) *versus* time (weeks). Virus half-life was then calculated using the equation virus half-life = (ln2)/k. Statistical analyses were performed on the linear regression model by ANOVA using the *F* statistic (Minitab release 16 software). The T_0 and T_1 values of a same series were also compared using a paired *t*-test, with $P < 0.05$ being statistically significant.

Meat viral load

The meat viral load was estimated at T_{10} or T_{max} by multiplying the normalized PRRSV RNA copies per mg by the total meat quantity given to 1 pig for the bioassay (around 500 g).

Phylogeny analysis

Phylogeny analysis was done using the nucleotide sequence of an amplified PRRSV ORF-5 region of NVSL. The RT-PCR was done using primer set P420 and P620 (24) and the One-Step RT-PCR Kit as described. The nested PCR was done using primer set 5FN and 5DN (25) and Platinum TAQ DNA Polymerase Kit (Invitrogen, Carlsbad, California, USA). The ORF-5 cDNA sequences were aligned using the neighbor-joining method with Clone Manager (Scientific & Educational Software, Cary, North Carolina, USA). An ORF-5 sequence of 544 bp was used for the alignment, which corresponded to bp positions 13811 to 14354 of NVSL. Genbank reference strains used for the alignment were: 17198-6 (EF442776.1), 2000-54471A (EU556182.1), 34075-NE (U66380.1), 98-6470-1 (AF339493.1), CH-1a (AY032626.1), FJ-1 (AY881994.1), HB-1 sh/2002 (DQ642048.1), IA-27 (EU758940.1), IAF-EXP91 (L40898.1), IAF-Klop (U64928.1), Ingelvac ATP MLV (DQ988080.1), Ingelvac RespPRRS MLV (AF066183.4), Lelystad (M96262.2), Lena (JF802085.1), MD-001 (AF121131.1), MN184 (EF442777.1), NADC-8 (AF396835.1), PA8 (AF176348.2), PrimePAC (AF066384.1), PRRSV0003749 (DQ477778.1), SDSU73 (EF442775.1), and VR-2332 (AY150564.1).

Results

PRRSV in pig meat

Overall, 1500 fresh meat samples were collected randomly at the PHBP over a 2-year period. All meat samples were screened for the presence of PRRSV using the RT-PCR targeting both American and European strains. Among meat samples, 16 (1.1%) were found presumptive positive for PRRSV with a typical amplified fragment size of around 303 bp, a very faint band for some samples. Presumptive positives were tested with distinct ORF-7 RT-PCR to confirm the positive results and to define the PRRSV genotypes. Out of 16 presumptive PRRSV positive cases, 11 were confirmed positive with at least a second set of ORF-7 primers. Those confirmed positives represented 0.73% of the overall meat tested. All confirmed positives were typical of the North American strains. From these, 9 were selected to further estimate the viral decay and to determine the infectivity of the stored meat at T_{max} by feeding piglets during bioassays. Two samples (ID 245 and 587) were not selected because they were positive for PCV type 2 in RT-PCR (data not shown). A non-confirmed and 2 RT-PCR negative meat samples were also kept for the viral decay study. During the study, 3 confirmed PRRSV positive meats were detected in April (3/98), 2 in September (2/120), 2 in October (2/225), 1 for each January (1/91), February (1/123), May (1/112), and November (1/97), and none during the months of March (0/86), June (0/120), July (0/194), August (0/184), and December (0/50).

Estimation of PRRSV load and viral decay in pig meat

To estimate viral decay, qRT-PCR was performed on meat subsamples maintained in the PHBP freezer and collected weekly for up to 15 wk (Table I). During this follow-up series, only 4 meat samples had an average PRRSV RNA concentration above the LOQ. For meats ID 715 and 1311, the number of viral RNA copies in almost all the subsamples could not be estimated (< LOQ) although specific RT-PCR products were detected for PRRSV (10/10 and 9/11, respectively) confirming their positive status. For sample 1424, only 3 out of 10 subsamples were positive and values were below the LOQ. No follow-up subsamples were positive for ID 574 and ID 597 from T_1 to T_{max}. Sample ID 340 and both negative controls remained negative throughout the storage period.

To estimate the variation of virus decay over time, a ratio of normalized viral RNA copies of each quantifiable subsample of a follow-up series was calculated for samples ID 84, 115, 319, and 1332 (Table I). No significant decay in viral RNA concentrations of PRRSV was observed during the storage period. Subsamples from ID 84 were tested in 3 separate experiments to estimate the inter-assay coefficient of variation (CV%) of the meat extract viral RNA copies/μL. It was estimated at 42%, showing significant variation from one qRT-PCR test to another for the same sample.

The meat viral load was estimated at T_{10} or T_{max}, based on the viral RNA copies detected per mg of meat (Table I). The extraction efficacy factor was found to be extremely low for the internal extraction control that was used. Only 7% of the input virus in the homogenized meat subsamples was recovered after RNA extraction of the muscle tissues. Recovery rates between 1% and 12% were also reported in previous studies using process control viruses with mouse norovirus, mengo, and hepatitis E virus (26). Numbers vary according to the method, the matrix, and the control virus. Pork meat usually has a high fat content that can interfere with nucleic acid extraction and inhibit the subsequent detection step. Nevertheless, low recovery yields are associated with high inter-assay variations. Based on these results, each piglet consumed approximately $10^{9.2}$ to $10^{9.8}$ viral RNA copies of PRRSV for every 500 g of meat it was fed. This range was estimated to be equivalent to $10^{5.0}$ to $10^{5.6}$ viral RNA copies per mL after extraction.

Experimental transmission

Results of PRRSV and antibody detection from pigs fed with PRRSV positive meat samples are summarized in Table II. Overall, a total of 18 piglets were fed in 5 different bioassays using 2, 3, 1, 2, and 1 positive PRRSV meat samples, respectively. Two piglets were fed with a non-confirmed PRRSV meat sample. A total of 9 control piglets were included in the bioassays. Meat samples were generally readily consumed after much chewing. Usually, all meat pieces were eaten by the pigs within 30 min to 2 h. At one occasion, the sample was smaller and the pigs receiving the sample were fed

Table I. Variations of porcine reproductive and respiratory syndrome virus (PRRSV) concentrations in selected meat samples stored at −20°C over time

Meat ID[a]	Weeks at −20°C[b]	Normalized PRRS viral RNA copies per mg													Follow-up positive results (T_1 to T_{max})
		T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{max}[b]	Average[c]	
84	15	$10^{4.8}$	$10^{4.5}$	$10^{4.1}$	$10^{4.5}$	$10^{4.3}$	$10^{4.5}$	$10^{4.7}$	$10^{4.5}$	$10^{4.7}$	$10^{4.4}$	$10^{4.4}$	$10^{4.1}$	$10^{4.5}$	11/11
115	13	$10^{3.7}$	$10^{4.0}$	$10^{3.9}$	$10^{4.0}$	$10^{4.4}$	$10^{3.8}$	$10^{3.6}$	$10^{3.5}$	+	$10^{3.7}$	$10^{3.5}$	+	$10^{3.9}$	11/11
319	11	$10^{3.5}$	$10^{3.7}$	$10^{3.5}$	$10^{3.2}$	+	$10^{3.8}$	$10^{3.9}$	$10^{3.5}$	$10^{3.4}$	$10^{4.1}$	$10^{3.7}$	$10^{3.9}$	$10^{3.7}$	11/11
340	11	0	0	NT	0	0	0	0	0	0	0	0	0	0	0/10
574	11	+	0	0	0	0	0	0	0	0	0	0	0	< LOQ	0/11
597	11	+	0	0	0	0	0	0	0	0	0	0	0	< LOQ	0/11
715	11	+	+	+	+	+	+	+	NT	+	+	+	+	< LOQ	10/10
1311	11	$10^{3.6}$	+	+	0	+	$10^{3.7}$	+	+	+	+	+	0	< LOQ	9/11
1332	11	$10^{3.9}$	$10^{3.5}$	$10^{3.7}$	$10^{3.5}$	$10^{3.7}$	+	$10^{3.8}$	$10^{4.0}$	$10^{3.8}$	$10^{3.7}$	$10^{3.8}$	$10^{3.7}$	$10^{3.7}$	11/11
1400*	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0/11
1414*	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0/11
1424	11	0	0	+	+	0	0	0	0	0	+	0	NT	< LOQ	3/10

[a] PRRSV negative control (*).

[b] The total period of time the meat was stored at −20°C (T_{max}).

[c] The average PRRS viral RNA copy per mg calculated from meat subsample collected from week 1 (T_1) to the end of the storage period (T_{max}) and normalized based on the RNA extract concentration.

+ — Positive samples that are detected but are below the limit of quantification in quantitative RT-PCR; < LOQ — Average value below the limit of quantification of $10^{3.5}$ RNA copies per mg; ID — meat identification number; NT — samples not tested.

with a total of 386 g instead of ~500 g (ID 715, Table II). During the 28-day observation period, all pigs appeared healthy. Positive transmission was observed with 5 confirmed PRRSV positive meat samples (5/9, 55.6%). Transmission in PRRSV confirmed positive cases was detected at 7 d post-exposure (DPE) by PCR analysis of the serum and throat samples and at 14 DPE by serology. None of the control piglets were shown to be infected. The meat viral load was an important factor for transmission. Oral transmission was detected with all meat samples (4/4) with quantifiable viral load. The pair of piglets fed with meat sample ID 1332 was found positive with both meat pieces stored at RT and at 4°C. Positive transmission was also detected with only 1 of the 2 piglets exposed to sample ID 1424. This sample remained non-quantifiable throughout the follow-up in the freezer and only a limited set of subsamples at T_2, T_3, and T_9 were found to be qRT-PCR positive (< LOQ). For this animal, viral transmission occurred only with the sample that was thawed at 4°C. The pair of piglets that was fed with meat sample ID 1311 was also apparently exposed to a lower virus load and was not infected. Average PRRSV RNA copies in meat sample ID 1311 was below the limit of quantification except at T_0 and T_5. The pair of piglets that consumed the non-confirmed positive meat sample ID 340 was not infected either.

Homology of strains

Strains from positive transmission bioassays were successfully sequenced and matched both before and after transmission (5/5). However, not all 11 PRRSV cases confirmed to be positive were successfully sequenced. The ORF-5 targeted sequence of 544 bp was obtained for 6 isolates only. Two isolates were only partially sequenced (ID 245 and 715). The quality of ID 587 ORF-5 virus sequence remains low. Two viruses could not be amplified by nested RT-PCR despite several attempts (ID 597 and 574). These difficulties could have been attributed to the low concentration of PRRSV RNA in meat samples (i.e., < LOQ) or strain variability. Based on the PRRSV ORF-5 sequence homology, 2 of the positive PRRSV transmissible meats (ID 319 and 1332) showed a close relationship (> 97%) with modified live virus Ingelvac RespPRRS MVL vaccine and the strains of lineage 5 over 544 bp (Figure 1) (27). The meat sample ID 715, confirmed positive but negative in the transmission bioassay, was also closely related to RespPRRS MVL vaccine type (98%) and was 100% homologous to meat sample ID 1332 over a smaller sequence stretch of 457 bp. Four confirmed positive PRRSV meats (ID 84, 115, 1311, and 1424) were related (~ 90%) to wild-type strains of lineage 1 such as MN184 and IAF-Klop (Figure 1). The meat ID 245 was also closely related to the lineage 1 genotype. The meat ID 245 ORF-5 sequence was closely related to meat 84 (100% over 478 bp). Sample IDs 245 and 715 were not included in the ORF-5 phylogram because it was not possible to cover the same ORF-5 region with these samples. The control strain used during the RT-PCR (NVSL) did not show a high homology with the detected sequences (< 90%). The current study confirmed the presence of 3 Ingelvac RespPRRS MVL related vaccine-type and 5 wild-type strains in confirmed PRRSV positive meats.

Discussion

The percentage of presumptive RT-PCR positive PRRSV meat samples was very similar to the ones reported previously in meat samples from Quebec (1.07% *versus* 1.2%) (6). However, only 11/16 were confirmed. In contrast, others have not found PRRSV by

Table II. Porcine reproductive and respiratory syndrome virus (PRRSV) detection in pigs experimentally fed with PRRSV positive meat samples

Pig	Meat ID	Meat weight (g)[b]	Estimated meat viral load (PRRSV copy)[c]	Bioassay (trial #)	°C[a]	RT-PCR/ELISA[d] (Days post exposure) −7	0	7	14	21	28
844-02	84	253/249	$10^{9.8}$	1	4	−/−	−/−	+/−	+/+	+/+	−/+
844-07	84	251/251	$10^{9.8}$	1	4	−/−	−/−	+/−	+/+	+/+	+/+
844-04	319	250/250	$10^{9.6}$	1	4	−/−	−/−	+/−	+/+	+/+	+/+
844-06	319	250/250	$10^{9.6}$	1	4	−/−	−/−	+/−	+/+	+/+	−/+
844-01	340	246/246	0	1	4	−/−	−/−	−/−	−/−	−/−	−/−
844-09	340	245/250	0	1	4	−/−	−/−	−/−	−/−	−/−	−/−
844-08	Ctrl	0/0	—	1	—	−/−	−/−	−/−	−/−	−/−	−/−
844-05	Ctrl	0/0	—	1	—	−/−	−/−	−/−	−/−	−/−	−/−
848-09	574	250/250	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-02	574	250/250	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-06	597	250/237	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-07	597	250/233	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-10	715	251/135	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-04	715	251/136	+	2	4	−/−	−/−	−/−	−/−	−/−	−/−
848-05	Ctrl	0/0	—	2	—	−/−	−/−	−/−	−/−	−/−	−/−
848-08	Ctrl	0/0	—	2	—	−/−	−/−	−/−	−/−	−/−	−/−
852-04	115	250/238	$10^{9.2*}$	3	4	−/−	−/−	+/−	NT	NT	+/+
852-05	115	251/239	$10^{9.2*}$	3	4	−/−	−/−	+/−	+/+	NT	+/+
852-01	Ctrl	0/0	—	3	—	−/−	−/−	−/−	−/−	−/−	−/−
874-04	1311	250/250	+	4	4	−/−	−/−	−/−	−/−	−/−	−/−
874-02	1311	250/250	+	4	RT	−/−	−/−	−/−	−/−	−/−	−/−
875-01	1424	250/250	+	4	4	−/−	−/−	+/−	+/+	+/+	+/+
874-03	1424	250/250	+	4	RT	−/−	−/−	−/−	−/−	−/−	−/−
874-01	Ctrl	0/0	—	4	—	−/−	−/−	−/−	−/−	−/−	−/−
875-02	Ctrl	0/0	—	4	—	−/−	−/−	−/−	−/−	−/−	−/−
879-03	1332	250/250	$10^{9.5}$	5	4	−/−	−/−	+/−	+/+	+/+	+/+
879-02	1332	250/250	$10^{9.5}$	5	RT	−/−	−/−	+/−	+/+	+/+	+/+
878-07	Ctrl	0/0	—	5	—	−/−	−/−	−/−	−/−	−/−	−/−
879-01	Ctrl	0/0	—	5	–	−/−	−/−	−/−	−/−	−/−	−/−

[a] The meat was stored at 4°C or room temperature (RT) for 24 and 48 h after storage at the PHBP.

[b] Each meat sample was divided in 2 equal parts The quantity of meat sample given on both days to each piglet.

[c] The total PRRS viral load in equivalent viral copies calculated using the ORF7 qRT-PCR results from aliquots of the meat homogenate stored at −20°C at T_{max}. Value calculated using concentration at T_{10} (*). Confirmed positive samples that are below the limit of quantification in quantitative RT-PCR(+).

[d] Serum test results from ORF7-RT-PCR genotype 2 assays and HerdCheck ELISA results either positive (+) or negative (−).

ID — meat identification number; NT — not tested.

RT-PCR in carcasses at slaughter (14). The prevalence, the meat sampling changes and the sensitivity of the RT-PCR assays could explain the PRRSV detection variation observed between studies. In addition, meat is a heterogeneous matrix composed of various elements such as fat, muscles, nerves, and vessels. In actively infected animals, PRRSV is not expected to be evenly distributed in meat but rather, located in residual blood (12,28). It is thus expected to be unequally distributed among subsamples collected to conduct the studies, leading to significant variations. In the current study, the levels of viral RNA were relatively low in general and close to the limit of detection of the molecular assays. The impact of assay sensitivity and low recovery yields on prevalence estimates is not negligible in such cases (19). The aging of the screening ORF7 primers is probably not a major issue based on their homologies with the reference PRRSV strains of the 2008 circulating lineages (27). Other factors associated with the sampling scheme could have influenced prevalence estimates, such as the type of meats, the handling of the carcasses before packaging, and the seasons during which the samplings were performed. Despite having taken the necessary precautions, samples collected at the PHBP could have been cross-contaminated in the processing line or during sample analysis. The confirmation steps used herein reduce the probability of overestimating the presence of the virus in pig meat and underestimating their transmission in the bioassays.

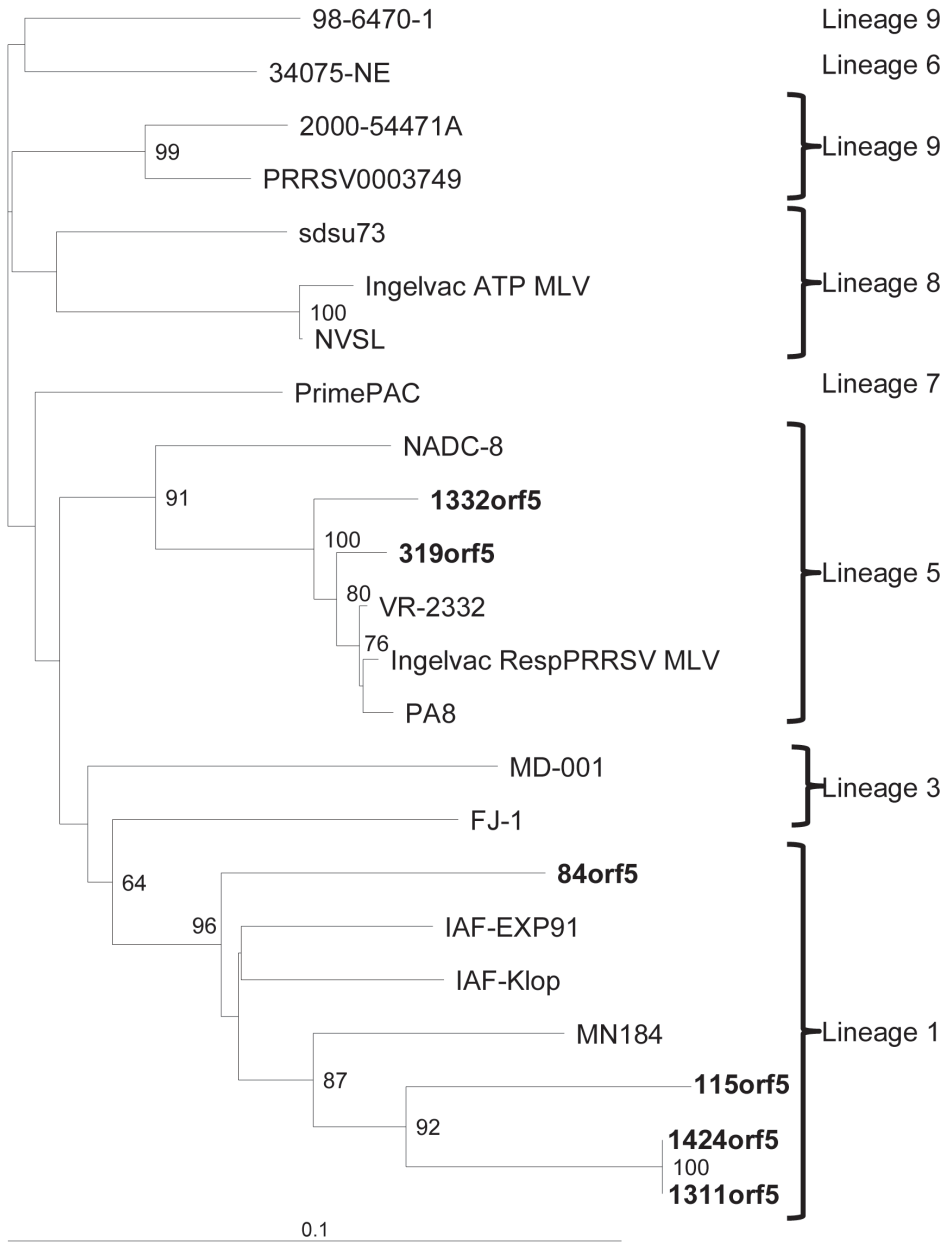

Figure 1. Phylogram of porcine reproductive and respiratory syndrome (PRRS) viral ORF-5 cDNA sequence and confirmed positive meat samples (in bold). The X-axis represents the percentage divergence. The numbered lineages follow the classification described previously (27). Internal labels represent the percentage of 1000 trees that support the clustering, only bootstrap values > 50% are shown.

No PRRSV positive meat was detected for 3 consecutive months during summer, although 33% of the meat samples were collected during those months. Although more sampling would be needed to confirm this trend, those results suggest that fewer pigs at the age of slaughter are infected with PRRSV or the level of infection is lower during summer. A study conducted by Larochelle et al (29) in Quebec reported that about 75% of field cases of PRRSV between 1998 and 2002 were submitted from November to April, suggesting a higher prevalence of disease during colder months. Other studies have also shown more frequent mechanical transmissions of PRRSV in cold weather than in warm weather (30,31). Considering that the stability of PRRS virions decreases when the temperature increases (12,32–34), these trends seem to confirm the lower prevalence of PRRSV during the summer months, suggesting a lower risk in oral transmission.

Similarly, the standard meat storage temperature at the packaging center might be too low to affect PRRS viral decay in frozen meat within the study time frame. The 15-week follow-up study was designed to provide insight on the virus integrity in meat throughout the period of time needed for transportation and storage during exportation overseas. No significant decay of viral RNA was detected in our study, but that could be the result of the subsamples

heterogeneity, the variations in inter-assays, and the poor extraction efficiency. It could also be the result of the study time frame and the RNA stability. Indeed, the data are compatible with the reported absence of PRRSV titer variation after 10 wk at −20°C in cell culture media (12), while others have reported a decrease in viral infectivity over time with storage temperature increase (−20°C to 30°C) in absence of viral RNA concentration variation (34,35).

Several studies have analyzed the oral transmission of PRRSV to pigs in order to assess the risk associated with swill feeding (36). This study is the first to report on the PRRSV RNA concentrations in pig meat collected at the PHBP, that are associated with positive transmission after storage in commercial conditions and thawing. For meat viral loads above 10^9 total PRRSV RNA copies/500 g of meat, the transmission rate was high (100%) but these samples were still relatively rare in the survey. The transmission rate of positive PRRSV meat samples was slightly lower than the one reported by Magar and Larochelle (6) (5/9 *versus* 7/11) in the same region. These results are surprisingly similar considering the differences between both studies, including the meat cuts, the storage temperature, and the variation in viral load.

The transmission rates observed with frozen packaged meat from the PHBP were relatively low when taking into account the prevalence of confirmed positive PRRSV meat products. The product of the prevalence and the transmission rate in this study (0.4% for 500 g) was in the same range as a model developed to describe the probability that meat imported from a country where PRRSV is present will contain an infectious dose of PRRSV after shipping (0.18% for 1 kg) (21). Several parameters, which could have influenced the transmission of PRRSV, were not considered in our study and require further investigation. For instance, scrap sizes (500 g *versus* 10 g) or disposal conditions (prolonged RT), which were not estimated or barely explored, should have an impact on the meat viral load and the transmission rates (37). Legislations, regulations, and training on swill feeding, must also be considered but were outside of the scope of this study. For example, swill feeding is not allowed in Canada. In addition, although the reversion of live attenuated vaccine strains to virulence under field conditions occurs (38), the inclusion of vaccine strains or vaccine strain derivatives in risk assessments requires balancing their benefits. In the current study, close to a third of the sequenced strains were related to vaccine types. These numbers are similar to the ones reported previously in Canada (6,7). The results indicate that the likelihood of meat from the PHBP contains wild strains of PRRSV and is infectious, is low. On the other hand, the elimination of vaccine strains from herds could require an equal amount of effort as the elimination of the wild type strains. The viral and clinical outcomes in piglets orally infected with pig meat and maintained in natural conditions, are still unknown.

In summary, low residual quantities of PRRSV are found in a small percentage of pig meat collected at the pork ham boning plant. The values observed were very similar to and/or slightly lower than the ones reported previously for the province of Quebec. No statistically significant degradation of the PRRSV RNA was observed at −20°C during 15 wk. Previously frozen PRRSV-positive meat was able to infect naïve pigs by oral exposure. Transmission was more efficient for the meat samples containing a quantifiable viral load, in the range of 10^9 genomic equivalents. However, the prevalence of these meat samples with a high PRRSV viral load remained low, especially when considering field strains only.

Acknowledgments

This research was made possible through the financial support of the Canadian Food Inspection Agency, the Canadian Pork Council, and Olymel. We would like to acknowledge Dr. Bianca Morel and her team for their assistance in the serological assays and sampling, Drs. Fatima Belayat and Yves Robinson for their assistance with autopsies, M. René Mineau for his support in the animal facilities, Ms Sylvianne Paul for her technical assistance, the CFIA Ottawa Fallowfield Laboratory and M. Steve Smith for providing SPF piglets, Dr. Carl Gagnon for consultation, and M. André Perron for revision of the manuscript.

References

1. Holtkamp DJ, Kliebenstein JB, Neumann EJ, et al. Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod 2013;21:72–84.
2. Music N, Gagnon CA. The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. Anim Health Res Rev 2010;11:135–163.
3. Niederwerder MC, Rowland RR. Is there a risk for introducing porcine reproductive and respiratory syndrome virus (PRRSV) through the legal importation of pork? Food Environ Virol 2017; 9:1–13.
4. Dea S, Gagnon CA, Mardassi H, Pirzadeh B, Rogan D. Current knowledge on the structural proteins of porcine reproductive and respiratory syndrome (PRRS) virus: Comparison of the North American and European isolates. Arch Virol 2000;145: 659–688.
5. Nelson EA, Christopher-Hennings J, Drew T, Wensvoort G, Collins JE, Benfield DA. Differentiation of US and European isolates of porcine reproductive and respiratory syndrome virus by monoclonal antibodies. J Clin Microbiol 1993;31:3184–3189.
6. Magar R, Larochelle R. Evaluation of the presence of porcine reproductive and respiratory syndrome virus in pig meat and experimental transmission following oral exposure. Can J Vet Res 2004;68:259–266.
7. Brar MS, Shi M, Ge L, Carman S, Murtaugh MP, Leung FC. Porcine reproductive and respiratory syndrome virus in Ontario, Canada 1999 to 2010: Genetic diversity and restriction fragment length polymorphisms. J Gen Virol 2011;92:1391–1397.
8. Murtaugh MP, Stadejek T, Abrahante JE, Lam TT, Leung FC. The ever-expanding diversity of porcine reproductive and respiratory syndrome virus. Virus Res 2010;154:18–30.
9. Karniychuk UU, Geldhof M, Vanhee M, Van Doorsselaere J, Saveleva TA, Nauwynck HJ. Pathogenesis and antigenic characterization of a new East European subtype 3 porcine reproductive and respiratory syndrome virus isolate. BMC Vet Res 2010;6:30.
10. Li B, Fang L, Guo X, et al. Epidemiology and evolutionary characteristics of the porcine reproductive and respiratory

syndrome virus in China between 2006 and 2010. J Clin Microbiol 2011;49:3175–3183.
11. Murtaugh MP, Genzow M. Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine 2011;29:8192–8204.
12. Bloemraad M, De Kluijver EP, Petersen A, Burkhardt GE, Wensvoort G. Porcine reproductive and respiratory syndrome: Temperature and pH stability of Lelystad virus and its survival in tissue specimens from viraemic pigs. Vet Microbiol 1994;42:361–371.
13. Frey ML, Landgraf JG, Schmitt BJ, Eernisse KA, Pearson JE. Recovery of porcine reproductive and respiratory syndrome virus from tissues of slaughter weight pigs. Second International Symposium on Porcine Reproductive and Respiratory Syndrome (PRRS) 1995:28.
14. Wang FI. Minimal residues of porcine reproductive and respiratory syndrome virus in pig carcases and boar semen. Proc Natl Sci Counc Repub China B 1999;23:167–174.
15. Murray N, Pharo H. Import risk analysis: Porcine reproductive and respiratory syndrome (PRRS) virus in pig meat [report on the Internet]. Wellington: Biosecurity New Zealand, Ministry of Agriculture and Forestry c2006 [updated 2006 July 25]. Available from: https://mpi.govt.nz/document-vault/2813 Last accessed May 5, 2017.
16. Neumann EJ, Morris RS, Sujau M. Analysis of the risk of introduction and spread of porcine reproductive and respiratory syndrome virus through importation of raw pigmeat into New Zealand. N Z Vet J 2007;55:326–336.
17. Steverink P. Transmission of porcine reproductive and respiratory syndrome virus through the oral uptake of infected porcine muscular tissue by naïve recipients [Report]. Lelystad, Netherlands: 2000.
18. van der Linden IF, van der Linde-Bril EM, Voermans JJ, et al. Oral transmission of porcine reproductive and respiratory syndrome virus by muscle of experimentally infected pigs. Vet Microbiol 2003;97:45–54.
19. European Food Safety Authority (EFSA). The probability of transmission of porcine reproductive and respiratory syndrome virus (PRRSv) to naïve pigs via fresh meat. EFSA J 2005;239: 1–85.
20. O'Neil BD. Re: Analysis of the risk of introduction and spread of porcine reproductive and respiratory syndrome virus through importation of raw pigmeat into New Zealand. N Z Vet J 2008; 56:48.
21. Pharo H, Cobb SP. The spread of pathogens through trade in pig meat: Overview and recent developments. Rev Sci Tech 2011;30:139–148.
22. Larochelle R, Magar R. Evaluation of the presence of porcine reproductive and respiratory syndrome virus in packaged pig meat using virus isolation and polymerase chain reaction (PCR) method. Vet Microbiol 1997;58:1–8.
23. Donadeu M, Arias M, Gomez-Tejedor C, et al. Using polymerase chain reaction to obtain PRRSV-free piglets from endemically infected herds. J Swine Health Prod 1999;7:255–261.
24. Cheon DS, Chae C. Restriction fragment length polymorphism analysis of open reading frame 5 gene of porcine reproductive and respiratory syndrome virus isolates in Korea. Arch Virol 2000;145:1481–1488.
25. Andreyev VG, Wesley RD, Mengeling WL, Vorwald AC, Lager KM. Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. Arch Virol 1997;142:993–1001.
26. Gentry-Shields J, Jaykus LA. Comparison of process control viruses for use in extraction and detection of human norovirus from food matrices. Food Res Int 2015;77:320–325.
27. Shi M, Lam TT, Hon C, et al. Phylogeny-based evolutionary, demographical, and geographical dissection of North American type 2 porcine reproductive and respiratory syndrome viruses. J Virol 2010;84:8700–8711.
28. Magar R, Robinson Y, Dubuc C, Larochelle R. Evaluation of the persistence of porcine reproductive and respiratory syndrome virus in pig carcasses. Vet Rec 1995;137:559–561.
29. Larochelle R, D'Allaire S, Magar R. Molecular epidemiology of porcine reproductive and respiratory syndrome virus (PRRSV) in Quebec. Virus Res 2003;96:3–14.
30. Dee S, Deen J, Rossow K, et al. Mechanical transmission of porcine reproductive and respiratory syndrome virus throughout a coordinated sequence of events during cold weather. Can J Vet Res 2002;66:232–239.
31. Dee S, Deen J, Rossow K, et al. Mechanical transmission of porcine reproductive and respiratory syndrome virus throughout a coordinated sequence of events during warm weather. Can J Vet Res 2003;67:12–19.
32. Benfield DA, Nelson E, Collins JE, et al. Characterization of swine infertility and respiratory syndrome (SIRS) virus (isolate ATCC VR-2332). J Vet Diagn Invest 1992;4:127–133.
33. Hermann J, Hoff S, Muñoz-Zanzi C, et al. Effect of temperature and relative humidity on the stability of infectious porcine reproductive and respiratory syndrome virus in aerosols. Vet Res 2007;38:81–93.
34. Zimmerman JJ, Jacobs AC, Hermann JR, et al. Stability of porcine reproductive and respiratory syndrome virus at ambient temperatures. J Vet Diagn Invest 2010;22:257–260.
35. Guarino H, Moura J, Cox RB, Goyal SM, Patnayak DP. Survival of porcine reproductive and respiratory syndrome virus in fresh pork. Acta Vet Hung 2014;62:257–263.
36. Hall W, Neumann E. Fresh pork and porcine reproductive and respiratory syndrome virus: Factors related to the risk of disease transmission. Transbound Emerg Dis 2015;62:350–366.
37. Brookes VJ, Hernández-Jover M, Holyoake P, Ward MP. Import risk assessment incorporating a dose-response model: Introduction of highly pathogenic porcine reproductive and respiratory syndrome into Australia via illegally imported raw pork. Prev Vet Med 2014;113:565–579.
38. Nielsen HS, Oleksiewicz MB, Forsberg R, Stadejek T, Bøtner A, Storgaard T. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J Gen Virol 2001;82:1263–1272.